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Enzyme-linked immunosorbent assay of changes in serum levels of growth hormone (cGH) in common carps (*Cyprinus carpio*)

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The aim of the present study was to purify the common native carp growth hormone (ncGH), produce monoclonal antibodies (mAbs) to common native carp growth hormone (ncGH), and further enhance the sensitivity of enzyme-linked immunosorbent assays (ELISA) for ncGH. Additionally, we investigated changes in serum ncGH levels in carps raised in different environmental conditions. The recombinant grass carp (*Ctenopharyngodon idella*) growth hormone was purified and used as antigen to immunize the rabbit. The natural ncGH was isolated from the pituitaries of common carp. SDS-PAGE and Western blot utilizing the polyclonal anti-rgcGH antibody confirmed the purification of ncGH from pituitaries. Purified ncGH was then used as an immunogen in the B lymphocyte hybridoma technique. A total of 14 hybridoma cell lines (FMU-cGH 1—14) were established that were able to stably secrete mAbs against ncGH. Among them, eight clones (FMU-cGH1—6, 12 and 13) were successfully used for Western blot while nine clones (FMU-cGH 1—7, 9 and 10) were used in fluorescent staining and immunohistochemistry. Epitope mapping by competitive ELISA demonstrated that these mAbs recognized five different epitopes. A sensitive sandwich ELISA for detection of ncGH was developed using FMU-cGH12 as the coating mAb and FMU-cGH6 as the enzyme labeled mAb. This detection system was found to be highly stable and sensitive, with detection levels of 70 pg/mL. Additionally, we found that serum ncGH levels in restricted food group and in the net cage group increased 6.9- and 5.8-fold, respectively, when compared to controls, demonstrating differences in the GH stress response in common carp under different living conditions.

common carp, growth hormone, monoclonal antibodies, ELISA, starvation, net cage

Growth hormone (GH) plays a crucial role in stimulating and controlling the growth, metabolism and differentiation of many cell types, by modulating mRNA and protein synthesis[1]. In fish, GH participates in almost all the major physiological processes in the body, including (1) the regulation of ionic and osmotic balance, (2) lipid, protein, and carbohydrate metabolism, (3) skeletal and soft tissue growth, (4) reproduction, and (5) immune function[2]. Some of these effects are brought about indirectly via insulin-like growth factors (IGF), which are produced in the liver and other peripheral tissues; other effects result from direct action on target cells[3,4]. GH receptors (GHRs) are expressed in many tissues, such as the liver, muscle, adipose tissue, bone, cartilage, and

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brain, with the highest levels of expression generally found in the liver[5−7].

Various assay methods, such as radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA), have been established to detect GH serum levels[8−11]. Although RIA is sensitive and specific, its use is limited by safety concerns of radioactive isotope contamination, as well as concerns regarding radioactivity attenuation. ELISA is a simple method, with no radioactivity concerns, however its use for GH quantitation has been limited by the lack of two or more monoclonal antibodies (mAbs) able to recognize different epitopes of GH molecule. To date, most ELISA systems have been based on the use of one monoclonal or polyclonal Ab. To our knowledge, no ELISA mAb system has been reported which utilizes two Abs for GH detection[12,13].

In the current study, 14 hybridoma cell lines, able to stably secrete mAbs against cGH, were established. Among them, two mAbs were selected to further develop a cGH-specific ELISA sandwich to detect native GH in carp. Our data suggested that serum cGH levels of common carps varied, depending upon living conditions of carp.

1 Materials and methods

1.1 Purification of recombinant grass carp GH

Medium (3 L) containing recombinant grass carp growth hormone (rgcGH) was prepared as previously described[14]. The medium was centrifuged (10 min, 4000×*g*) and the supernatant was ultrafiltrated utilizing a 50 kDa membrane (Millipore) and concentrated to 200 mL with a 3-kDa ultrafiltration membrane (Millipore). The protein solution was adjusted to pH 7.0 with Tris, and then loaded onto a DEAE Sepharose Fast Flow column (Amersham Biosciences), which was pre-equilibrated in 20 mmol/L pH 7.0 phosphate buffer. The column was washed with the same buffer and the binding proteins were eluted with a linear gradient of 0 to 1 mol/L NaCl in the presence of 20 mmol/L pH 7.0 phosphate buffer. The eluate was analyzed on a 12% SDS-PAGE gel. Fractions containing rgcGH were desalted with a Sephadex G-25 column (Amersham Biosciences), pre-equilibrated with 10 mmol/L NH_4HCO_3 and then lyophilized. The purified rgcGH was used as the antigen to immunize rabbits.

1.2 Preparation of polyclonal antibody in rabbits

Purified rgcGH was prepared in complete Freund's adjuvant (FCA), and then injected subcutaneously at 20 sites on the thigh of two New Zealand white rabbits. The first booster injection was given with rgcGH prepared in incomplete Freund's adjuvant (FIA) 15 days later, followed by booster injections every 15 days thereafter. Bleedings were performed 45 days after the initial immunization and continued every 15 days. The serum was separated and the titer was measured by ELISA using purified rgcGH as the detection antigen, with serum from non-injected rabbits used as control. High-titer antibodies containing serum were stored at –20℃.

1.3 Preparation and purification of the native common carp growth hormone (ncGH)

Frozen pituitaries (approximately 5 g) were homogenized in extraction buffer (50 mL) containing 50 mmol/L Tris·HCl pH 9.0, 500 mmol/L NaCl, 1 mmol/L PMSF, 1% streptomycin sulphate, followed by the addition of 1 mol/L $CaCl_2$ (50 mL) and 10% Dextran sulphate (2 mL). The crude solution was centrifuged at low speed (15 min, 4℃, 200 r/min) followed by high speed centrifugation (10 min, 4℃, 10000×*g*) to obtain the final supernatant, which was then loaded onto a Sephadex G-25 column (Amersham Biosciences), pre-equilibrated with a solution of 20 mmol/L Tris·HCl pH 7.5, 250 mmol/L NaCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$ and 1 mmol/L $MnCl_2$ and eluted by the same buffer. The eluate was loaded onto the ConA Sepharose column (Amersham Biosciences) to rescue additional hormones, and the fraction containing GH was eluted with a buffer containing 20 mmol/L Tris·HCl pH 7.5, 500 mmol/L NaCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$ and 1 mmol/L $MnCl_2$. The solution was concentrated utilizing a 5 kDa ultrafiltration membrane (Millipore) and then loaded onto a Sephadex G-25 column pre-equilibrated with a solution containing 20 mmol/L Tris·HCl pH 8.0. A desalting solution was then loaded onto a DEAE Sepharose Fast Flow column (Amersham Biosciences) pre-equilibrated with 20 mmol/L Tris·HCl pH 8.0. Binding proteins were eluted with a linear gradient, (0 to 1 mol/L NaCl), in the presence of 20 mmol/L Tris·HCl pH 8.0. Fractions were then analyzed by 12% SDS-PAGE and those fractions containing ncGH were mixed with equal volumes of 3 mol/L $(NH4)_2SO_4$ and loaded onto a Phenyl Sepharose 6 Fast Flow column (Amersham Biosciences) pre-equili-

brated with 1.5 mol/L $(NH4)_2SO_4$. Protein fractions were eluted with a linear gradient (1.5 to 0 mol/L $(NH_4)_2SO_4$) in the presence of 20 mmol/L Tris·HCl pH 8.0 and were once again analyzed by 12% SDS-PAGE. The ncGH fraction was concentrated to a volume of about 1 mL. The crude ncGH was purified utilizing a Sephacryl S-100 High Resolution column, pre-equilibrated with 10 mmol/L NH_4HCO_3. Purified ncGH was then subjected to 12% SDS-PAGE and Western blot analysis with the polyclonal anti-rgcGH antibody.

1.4 Production of hybridomas

Female BALB/c mice (8 weeks old) were immunized with 20 μg ncGH in FCA by subcutaneous (s.c.) injection. Subsequent s.c. immunizations were carried out twice with 20 μg ncGH in FIA and intra-peritoneal (i.p.) injections, without adjuvant respectively, at 3-week-intervals. Mice were bled from caudal vein 10 days after the third immunization and anti-serum titers were determined by indirect ELISA. Immunized mice were boosted with 20 μg of antigens by i.p. injection. Splenocytes from immunized mice and SP2/0 myeloma cells, cultured in RPMI 1640 containing 20% fetal calf serum (FCS), were fused, three days later, in the presence of PEG (MW4000, Merk, Germany). Positive hybrids were screened by ELISA and subcloned four times by limiting dilution. Monoclonal antibodies were produced either from supernatants of the hybridoma culture or from ascites of BALB/c mice in which hybridomas had been injected intraperitoneally. Ig isotypes were identified using an Isotype kit (Sigma, M-5907). Epitope specificities of mAbs to cGH were analyzed by competitive ELISA.

1.5 Indirect ELISA

In order to screen Abs reacting with cGH, 96-well plates (NUNC, Nagel Inc., Roskilde, Denmark) were coated with 5 μg/mL of cGH in coating buffer (0.05 mol/L carbonate/bicarbonate buffer, pH 9.5) and incubated (4℃, overnight). Plates were washed (3×) with 0.01 mol/L PBS / 0.05% Tween-20 and blocked with a 0.1% BSA solution. Various dilutions of immunized rabbit or murine sera or supernatants from hybridoma cultures were then added to the wells. Plates were incubated (1 h, 37℃) and washed (3×), then incubated with 100 μL of horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse antibody (Vector, USA), and diluted 1/5000 in PBS (1 h, 37℃). Plates were then washed (3×) and 100 μL of 2,2′-azobis-3-ethylbenzthiazoline-6-sulfonic (ABTS, Vector, USA) was added to each well prior to reading (410 nm) with an automatic plate reader (Bio-Rad).

1.6 Western blot

Purified ncGH was denatured, subjected to SDS-PAGE then transferred onto PVDF membranes (BioRad), and blocked (4℃, overnight) in TBS (20 mmol/L Trizma, 0.5 mol/L NaCl, pH 7.5) containing 5% non-fat milk. Membranes were incubated (room temperature, 2 h) with mAbs against ncGH. After washing, membranes were incubated with HRP-labeled goat anti-mouse Ig diluted in antibody buffer (1∶1000, 2 h). An enhanced Chemiluminescence (ECL, Roche, USA) kit was used to detect bands.

1.7 Immunohistochemistry

Sections from carp pituitary (20 μm) were used in immunohistochemical analysis of ncGH expression. Several Ab dilutions were tested to find the optimal staining concentration. The staining procedure, without protease treatment, has been previously described. Briefly, sections were washed (20 min) in 0.01 mol/L PBS, pretreated with methanol-H_2O_2 to remove endogenous peroxidase, rinsed in 0.1 mol/L PBS and incubated with goat serum (30 mL/L, 40 min). Samples were then incubated with primary mouse anti-ncGH antibody (1∶200, 25 μg/mL, 4℃, 24 h) followed by secondary biotin-labeled sheep anti-mouse IgG (1∶200, room temperature, 3 h, Vector, USA). Sections were subsequently incubated with avidin-biotin peroxidase complex (ABC Vector, USA, 1∶100 dilution, room temperature, 1 h), then washed (3×, 10 min). A colorimetric reaction utilizing diaminobezidin (DAB) was used to develop the sections. Negative control tissue sections utilized anti-staphylococcus enterotoxin D (SED) mAb as the primary Ab.

1.8 Purification, HRP-conjugating of mAbs and sandwich ELISA

Selected mAbs were purified from mouse ascites fluids. IgG was purified from ascites fluid utilizing a HiTrap protein G column (Amersham Biosciences). Purified mAbs were conjugated with HRP using peroxidase, as previously described[15]. In order to detect ncGH, a sandwich ELISA was developed using FMU-cGH12 as

the coating mAb and FMU-cGH6 as the enzyme-labeled mAb. In brief, 100 μL of anti-cGH mAb (5 μg/mL in 0.05 mol/L carbonate/bicarbonate buffer, pH 9.5) was added to each ELISA plate well (Nunc. Maxisorp), incubated overnight (4℃) and washed (3×) with 0.01 mol/L PBS containing 0.1% (*v*/*v*) Tween-20 (PBS/Tween). Plates were then blocked with a 0.1% BSA solution. Standard ncGH serial dilutions with PBS containing 0.1% BSA and 0.1% (*v*/*v*) Tween-20 or serum samples of carp or grass carp were added to the wells and incubated (1 h, 37℃). After extensive washing with PBS containing 0.1% (*v*/*v*) Tween-20 (PBS/Tween), wells were incubated (1 h, 37℃) with FMU-cGH6 conjugated with HRP and diluted in PBS containing 3% PEG. Wells were washed once again (3×) and 100 μL of ABTS substrate was added to each well. Plates were read at 410 nm on a microplate reader. The coefficient variation of inter-assay and intra-assay was measured respectively. Additionally, levels of prolactin, thyroid-stimulating hormone, follicle stimulating hormone, and luteinizing hormone were also measured.

1.9 Fish and experimental design

Farm-raised common carps (one year old, n=45) were divided randomly into three groups: group 1 (control, n=15) was transferred into a 10×10×0.8 m^3 pool; group 2 (Net cage, n=15) was kept in a 31.5×0.8 m^3 net cage, within the same pool as group 1. Both groups 1 and 2 received normal amounts of food. Group 3 (Food restriction, n=15) was placed in a pool similar to that of group 1, but was not fed artificially. Three months later, blood samples of each group were collected from the caudal vasculature, kept on ice and later centrifuged (3000×g, 4℃, 5 min) to obtain serum samples which were then examined by sandwich ELISA system.

1.10 Statistical analysis

Serum GH levels were analyzed using a Student-Newman-Keuls-q test. Average serum GH levels between different groups were expressed as $x \pm sd$; $P<0.05$ was considered significant.

2 Results

2.1 Identification of purified ncGH

A total of 18 mg of the target protein was purified from pituitaries. Western blot analysis utilizing a polyclonal Ab against rgcGH confirmed that the 23 kDa protein identified was ncGH (Figure 1). The ncGH purification rate was 87%.

2.2 Production of mAbs specific for ncGH

A total of 14 hybridoma cell lines were established which could stably secrete mAbs against ncGH (FMU-cGH 1－14). In almost all cages, the Ig subclass of mAbs secreted by the hybridomas was IgG1 (κ), with the exception of FMU-cGH 4 and FMU-cGH 14, which secreted IgG2b (κ). Titers of ascites fluid containing mAbs ranged between 10^{-7} and 10^{-5}, as measured by indirect ELISA. Secreted mAbs from FMU-cGH 1－6, 12 and 13 were successfully used for Western blot, and FMU-cGH 1－7, 9 and 10 were used in fluorescent staining and immunohistochemistry. Western blot analysis using specific mAbs further confirmed that the molecular weight of ncGH was 23 kDa (Figure 2). Immunostaining data localized ncGH staining to the cytoplasm, as membrane and nuclei remained immunonegative (Figure 3).

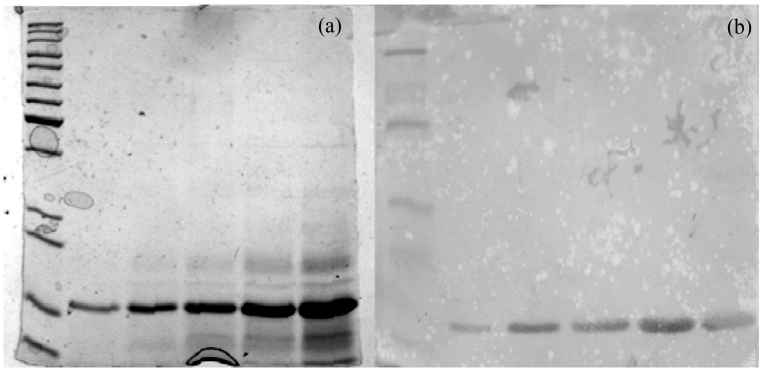

Figure 1 Identification of ncGH purified from common carp pituitaries (87% purification rate) under denaturing conditions. (a) SDS-PAGE of purified ncGH; (b) Western blot analysis of ncGH, utilizing a polyclonal Ab against rgcGH. Lane 1, molecular marker; lanes 2－6, representative samples at different concentrations.

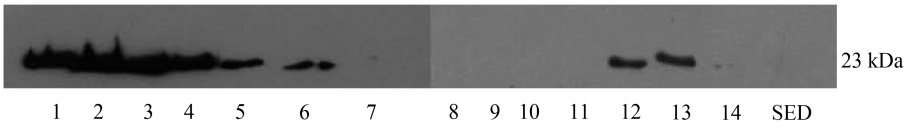

Figure 2 Identification of mAbs able to react with ncGH under denaturing conditions. Lanes 1－14, FMU-cGH 1－14, respectively.

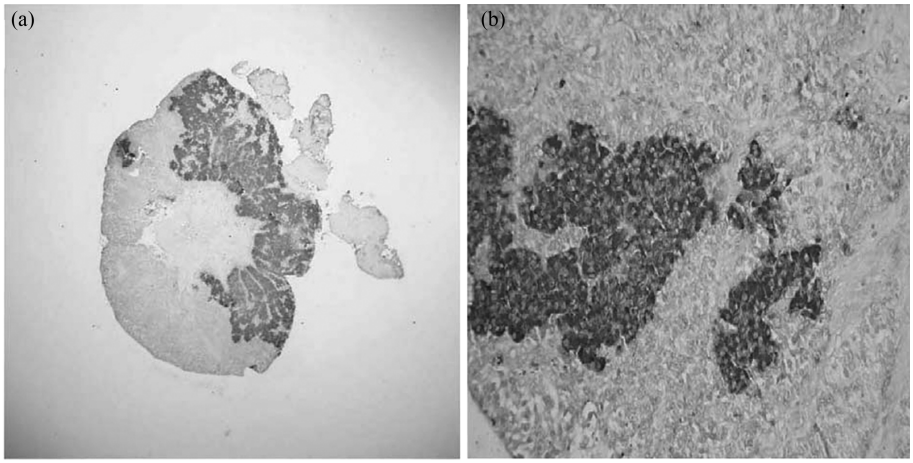

Figure 3 Distribution of ncGH in carp pituitary. A positive signal was seen mainly in the cytoplasm. (a) Whole pituitary sagittal section (40×); (b) pituitary sagittal cross-section (400×).

2.3 Development of ELISA for ncGH quantitation

Epitope mapping by competitive ELISA suggested that mAbs recognized five different epitopes. The first was recognized by FMU-cGH 1, 3, 8, 12 and 13; the second by FMU-cGH 6 and 7 and the third by FMU-cGH 9 and 11. FMU-cGH 4 and 10 recognized unique epitopes (Figure 4). A sandwich ELISA for detecting ncGH was developed using FMU-cGH12 as the coating mAb and FMU-cGH6 as the enzyme-labeled mAb. As seen in Figure 5, the ELISA system was very sensitive, with a limitation of 70 pg/mL. The inter-assay and intra-assay coefficient variation was 7% and 5%, respectively. The ELISA system was also able to detect GH concentrations in grass carp. However it was not able to detect other substances existing in the pituitary, such as prolactin, thyroid stimulating hormone, follicle stimulating hormone, and luteinizing hormone (n=3 independent experiments).

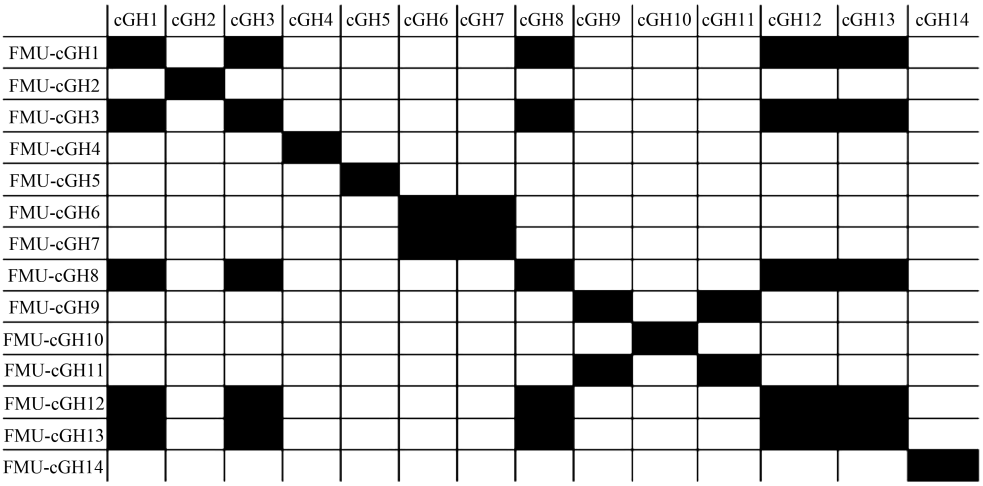

Figure 4 Epitope mapping by competitive ELISA. Both mAbs recognized the same epitope.

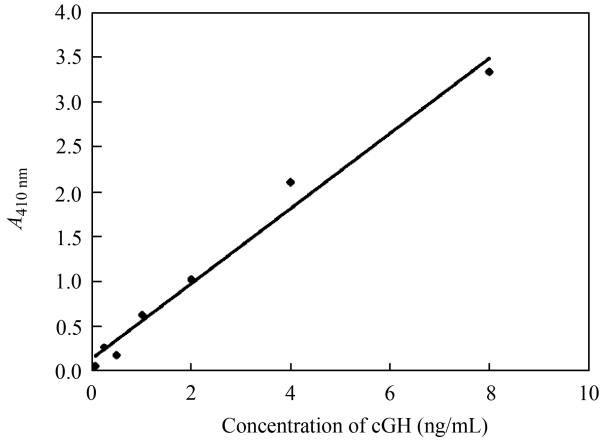

Figure 5 Sandwich ELISA standard curve for detecting ncGH. The detection limit was approximately 70 pg/mL (R^2=0.9827).

2.4 Elevated serum GH levels in carps subjected to food restriction or to net cage living conditions

The average serum ncGH levels of group 1 (control), group 2 (net cage), and group 3 (food restriction) were 0.73±0.74, 4.21±4.41 and 5.04±5.04 ng/mL, respectively. Compared with the control group, serum ncGH levels of common carp living in a net cage or subjected to food restriction increased 5.8 fold (P<0.05) and 6.9 fold (P<0.05), respectively. As shown in Figure 6, of the 15 fish in the control group, only four had serum ncGH levels >1.5 ng/mL of ncGH, with 2.2 ng/mL recorded as the highest serum ncGH level. Contrarily, fish in the net cage (n=11) and in the food restriction group (n=9) had serum cGH levels $\geqslant$1.5 ng/mL, with the highest levels of serum cGH measured at 16.0 and 14.0 ng/mL respectively (Figure 6).

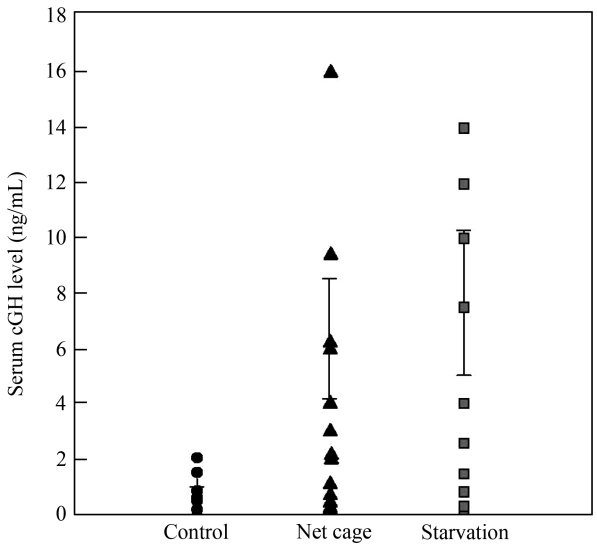

Figure 6 Serum growth hormone levels of different groups (x±sd). Of the 15 fish in the control group, only 4 displayed ncGH serum levels $\geqslant$ 1.5 ng/mL, while in the net cage and restricted food groups, serum ncGH levels greater or equal to 1.5 ng/mL were noted in 11 and 9 fish, respectively.

3 Discussion

In teleosts, GH, one of the pituitary hormones, is essential for the maintenance of growth. In our study, we produced a set of mAbs against ncGH, and confirmed that eight of the cell clones could be used in Western blot while nine clones could be used in both fluorescent staining and immunohistochemistry. Furthermore, we found that these mAbs recognized five different epitopes on the ncGH molecule. Based on the results we got above, a sensitive sandwich ELISA system with the limitation of 70 pg/mL against ncGH was developed successfully.

The development of GH isolation and purification, and the preparation of an antiserum to GH from immunized rabbits, a radioimmuno assay (RIA) was successfully developed, in 1983, to detect pituitary or serum GH of carp and goldfish (*Carassius auratus*)[16]. The detection limitation was 5 ng/mL. Soon afterwards, a RIA assay for detecting GH of teleosts, such as salmon (*Oncorhynchus keta*), chinook salmon (*Oncorhynchus tshawytscha*), European eel (*Anguilla anguilla*), and African catfish (*Arius africanus Günther*) was also developed[8−11]. However, this system had obvious limitations, such as radioisotope contamination and the short half-life of labeled tracers.

One of the advantages of the ELISA method is that it avoids potential radioactive contamination. The first sandwich ELISA kit for measuring oncorhynchid GH using mAbs was reported in 1991[12]. While this ELISA recognized GH in pituitary extracts or plasma of rainbow trout (*Oncorhynchus mykiss*), coho salmon (*Oncorhynchus kisutch*), and Chinook salmon, it did not recognize GH in carp, goldfish or other important teleosts. Additionally, it was less than 1.56 ng/mL. A new competitive ELISA to measure plasma GH levels in channel catfish was reported in 2003[17]. In this system, plates were coated with 25 μg of channel catfish (*Ictalurus punctatus*) GH in carbonate buffer. The standard hormone or plasma samples were then mixed with rabbit anti-cfGH, followed by the addition of HRP-labeled goat anti-rabbit antibody.

Specificity and stability are well-established advantages to using an ELISA two-mAb system that recog-

nizes different epitopes on the same molecule. For we know, the ELISA system we developed for detecting ncGH was the first one basing on two monoclonal antibodies which recognize different epitopes on the ncGH molecule. Besides specificity and stability, the ELISA displayed a very high sensitivity with the detection limitation of 70 pg/mL, which enabled us to further investigate the changes of serum ncGH levels in common carps living in different conditions.

Previous research has suggested that the stress response in teleost fish is, in many ways, similar to that of terrestrial vertebrates[18−20]. Growth hormone is one of the indicators of the teleost stress response. In the current study, we found a 6.9-fold increase in serum ncGH levels in the restricted food group and a 5.8-fold increase in the net cage group, compared to control. These data are consistent with previous reports in other fish living under similar conditions, such as salmon, rainbow trout, Oncorhynchus mykiss, European eel, and black seabream (*Acanthopagrus schlegeli*)[9,21−23].

In conclusion, the ELISA system we developed for detecting ncGH was very sensitive, stable and easy to perform since we selected monoclonal antibodies with high affinity and recognizing different epitopes of cGH from a number of monoclonal antibodies against ncGH we raised. This ELISA enabled us to compare serum ncGH levels in carps lived in different stress conditions. Compared with the control, serum ncGH level significantly increased in carps living either in starvation condition or in net cage. These novel mAbs to ncGH may be useful in future studies of ncGH, to further elucidate the relationship between GH and other hormones and the dynamic expression of cGH in the potential development of GH gene-transgenic carps.

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